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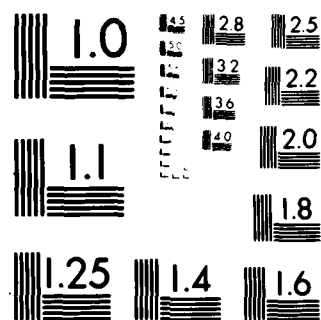
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IMMOBILIZED ENZYMES FOR AUTOMATED ANALYSES

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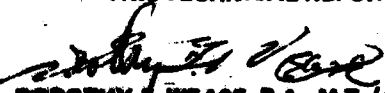
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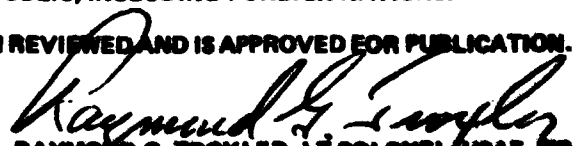
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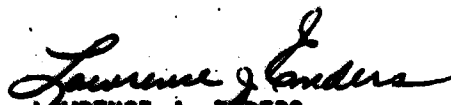
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20. ABSTRACT (Continued)

specific surface areas for immobilization, 39,000 to 150,000 cm^2/g of carrier.

The methods were applied to study of immobilization of glycerol dehydrogenase (GD). Three distinct types of immobilization processes were analyzed: a) irreversible physical adsorption to nylon 6,6; b) adsorption to charged amine-glass; and c) dialdehyde-linked covalent coupling to amine-glass. Adsorption to nylon (process a) indicated the importance of nicotinamide adenine dinucleotide (NAD) during immobilization. With no NAD present during coupling, ~6-7% of the bound enzyme retained its activity, while with 1.5 $\mu\text{mole/ml}$ NAD more than 50% of the bound enzyme was active. The total amount of GD bound, ~0.02 $\mu\text{g}/\text{cm}^2$, however, was too small for scheme (a) to be of practical interest.

Amine carriers were produced by treatment of glass microfiber filters with gamma-amino-propyl-tri-ethoxy-silane (GAPTES). This typically yielded 6.5-10¹⁶ surface amine groups per cm^2 . Using one type of the silane coupling agent, surface activity saturated at about 110-120 μg of active enzyme per carrier, for both direct and dialdehyde-coupled enzyme. This corresponded to ~50% of measured bound protein for the directly coupled amine glass carriers and to about 30% of firmly bound protein for the dialdehyde-linked enzyme.

Retention of activity after room temperature storage was dependent on complete exclusion of air from filter carriers. The directly coupled enzyme lost activity gradually in time at about 15% per week, while for the glutaraldehyde-coupled enzyme carriers it was possible to retain an activity equivalent to 100 to 110 μg of active enzyme over a 5-week period with storage in between assays at room temperature. The retained surface activity was equivalent to 3500 μg of active enzyme/g of carrier or 0.09 $\mu\text{g}/\text{cm}^2$ of carrier surface. With stable surface activity at nearly 0.1 $\mu\text{g}/\text{cm}^2$, the method would seem to have application to development of reactor tubes for glycerol quantitation in automated clinical chemistry analyzers.

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SUMMARY

The purpose of the study was to develop methods for analyzing enzyme immobilization processes with carrier materials which are suitable for use in automated clinical chemistry analyzers. The program focused on a particular application, immobilization of glycerol dehydrogenase (GD). Immobilization methods were analyzed in terms of:

- a) amount of bound enzyme protein
- b) surface activity
- c) quantity of "activating" chemical groups on the carrier surface
- d) total available carrier surface area.

A key feature of the program was the use of carrier materials in the form of microfibers or microspheres so as to present very large specific surface areas for immobilization (39,000 to 150,000 cm²/g of carrier).

Severe problems with both stability and activity of the free enzyme were dealt with prior to beginning the immobilization studies. The enzyme has been described (4) as very unstable at room temperature, even for periods of a few minutes, and it is generally held near 4°C until just before use. We found that stability was dependent primarily on oxidation; enzyme solutions stored with 1.5 μ mole/ml nicotinamide adenine dinucleotide (NAD) at pH 7 without air were stable for at least several days at room temperature (20°C).

Commercially available enzyme was used in the study. It was necessary to evaluate GD preparations from several sources in order to find a preparation which was suitable in terms of both activity and purity as determined by electrophoresis. Enzyme kinetics were briefly investigated. Michaelis constants K_m for glycerol and NAD substrates were determined and were in close agreement with values obtained by others. Reaction velocity was found to increase with temperature at a rate of about 5%/°C.

Three distinct types of immobilization processes were analyzed: a) irreversible physical adsorption to nylon 6,6; b) adsorption to charged amine glass; and c) dialdehyde-linked covalent coupling to amine glass. Adsorption to nylon (process a) indicated the importance of NAD during immobilization. With no NAD present during coupling, approximately 6-7% of the bound enzyme retained its activity, while with 1.5 μ mole/ml NAD more than 50% of the bound enzyme was active. The amount of bound enzyme was the same for

both cases, and the GD coupled with NAD was thus an order of magnitude more active. The total amount of GD bound, approximately $0.02 \mu\text{g}/\text{cm}^2$, however, was too small for scheme (a) to be of practical interest.

Amine carriers were produced by treatment of glass microfiber filters with gamma-amino-propyl-tri-ethoxy-silane (GAPTES). This typically yielded $6.5-10^{16}$ amine groups per cm^2 of carrier. Enzyme coupling was found to be dependent on the source of the GAPTES. Using one type of the silane coupling agent, surface activity saturated at about $110-120 \mu\text{g}$ of active enzyme per carrier, for both direct and dialdehyde-coupled enzyme. This corresponded to approximately 50% of measured bound protein for the directly coupled amine glass carriers and to about 30% of firmly bound protein for the dialdehyde-linked enzyme.

Retention of activity after room temperature storage was dependent on complete exclusion of air from the filter assembly. The directly coupled enzyme lost activity gradually in time at about 15% per week, while for the glutaraldehyde-coupled enzyme carriers it was shown to be possible to retain an activity equivalent to 100 to $110 \mu\text{g}$ of active enzyme over a 5-week period with storage in between assays at room temperature. This finding is one of the principal results of the program. The retained surface activity was equivalent to about $3500 \mu\text{g}/\text{g}$ or $0.09 \mu\text{g}/\text{cm}^2$ of carrier.

The second source of GAPTES yielded even higher initial surface activities. After exposure to enzyme solutions at $0.5 \mu\text{g}/\text{ml}$ or greater, the surface activity equivalent was approximately $200 \mu\text{g}$ per filter. However, a method for stabilizing the bound enzyme over time was not found for this second group of carriers.

The 9-month study demonstrated the feasibility of quantitatively analyzing the immobilization process, and a workable method for immobilizing glycerol dehydrogenase to amine-glass was delineated with stable surface activity at nearly $0.1 \mu\text{g}/\text{cm}^2$. The method would seem to have application to development of reactor tubes for glycerol quantitation in automated clinical chemistry analyzers.

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IMMOBILIZED ENZYMES FOR AUTOMATED ANALYSES

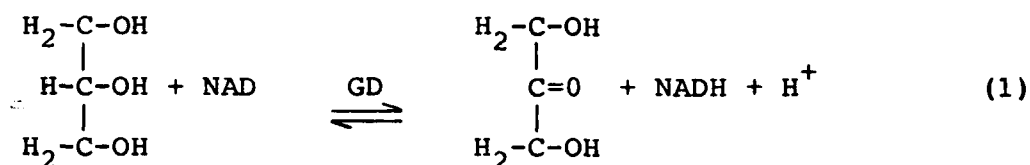
INTRODUCTION

Immobilization of enzyme to a carrier surface in a clinical chemistry analyzer enables catalysis of the analyzer reactions with retention of active enzyme by the system for subsequent reuse. When a large number of samples are processed, this can enable enormous savings in cost, as well as improvement in reproducibility.

Immobilization involves attachment of enzyme macromolecules to a surface with retention of catalytic activity. The surface may first be "activated" to facilitate bonding of enzyme molecules. The mode of attachment may be via irreversible adsorption, covalent chemical coupling, or entrapment in a gel matrix. All of these methods have been applied to immobilization to several enzymes of relevance to clinical chemistry. However, no universally applicable coupling scheme exists, and many of the schemes are recipe-like in nature. Often no data are given on amount and activity of bound enzyme per unit surface of carrier, data which are essential to practical utilization in a clinical analyzer or other system. Furthermore, the number of enzymes which have been immobilized to materials suitable for direct use in a flow system is very limited.

The present feasibility study was aimed at developing methods for quantitatively analyzing enzyme immobilization processes in general, with concentration on a particular application: measurement of glycerol using immobilized glycerol dehydrogenase. Several coupling methods, encompassing both physical adsorption and chemical coupling, were evaluated with emphasis on quantifying: a) the amount of irreversibly bound protein, b) the activity of the bound enzyme, c) number of "activated" surface chemical groups where such are required for immobilization, and d) total available surface area of the carrier. To readily carry out these measurements, carriers with very large specific surface areas are desirable so as to bind macroscopic amounts of enzyme. For this reason carriers in the form of microfibers or microspheres with specific surface areas in the range of 39,000 to 150,000 cm²/g of material were utilized. The carrier material was in all cases one which is readily available in the form of tubing so that each immobilization scheme could be directly implemented in a flow system. The use of a tubing material in large surface form results in an increase of several orders of magnitude in the amount of bound enzyme over that which would be possible using the same carrier in macroscopic form. Standard chemical methods can then be readily used to assay for amount and activity of bound enzyme, and for surface-active coupling groups.

Immobilization of glycerol dehydrogenase (GD) is of interest since GD provides a 1-step enzymatic quantitation of free glycerol, which constitutes a necessary phase in triglyceride determination.



This enzyme is not currently in wide use for this application. GD is expensive and thought to be relatively unstable. Most papers describing its application to clinical chemistry recommend storage at 4°C until just prior to use, and caution that loss of activity may occur in just 10 minutes at room temperature (4). Commercially available GD with the highest specified activity is 25 U/mg of protein, while enzymes which have been successfully immobilized have much higher intrinsic activity, e.g., lactate dehydrogenase typically 500 U/mg, hexokinase typically 140 U/mg (1). Considerations of stability and activity clearly indicate that immobilization of GD represents a substantial challenge. However, its direct applicability to 1-step enzymatic measurement of glycerol with the possibility retrofit to existing clinical chemistry analyzers provides rationale for this particular focus of the program.

PROCEDURE AND METHODOLOGY

Three coupling methods for GD immobilization were evaluated during the 9-month feasibility study. The program included:

- a) Characterization of the free enzyme with emphasis on identifying factors which maintain stability at room temperature and characterization of commercially available enzyme preparations.
- b) Selection of carrier materials and production of carriers in large surface-area form.
- c) Preparation and characterization of surface-active groups for coupling.
- d) Carrying out the immobilization processes.
- e) Measurement of irreversibly bound protein.
- f) Measurement of activity of the immobilized enzyme.
- g) Storage of the immobilized enzyme with periodic reassay of activity over time.

Each of these program elements is discussed below:

Characterization of Free Enzyme

Glycerol dehydrogenase is a product of the bacteria *Aerobacter aerogenes* which is separated from culture medium and purified

according to procedures first described by Burton (4). Preparation of the enzyme was beyond the scope of the study, and commercial preparations were used. Four of these were evaluated for activity using the protocol described in the "Measurement of Enzymatic Activity" section on p. 15. Commercial sources and specified activities (international units/mg of protein) were as follows:

PL Laboratories (Milwaukee, Wis.)	25 U/mg
Sigma Laboratories (St. Louis, Mo.)	4-5 U/mg
Worthington Biochemical (Freehold, N.J.)	8-10 U/mg
Boehringer Mannheim Biochemical (Indianapolis, Ind.)	25 U/mg

As discussed in the "Results" section on p. 16, only the Boehringer Mannheim preparation was satisfactory. The purity of this preparation and the Sigma preparation were characterized by cellulose acetate electrophoresis. Migration times were 20 minutes at 200 V; bands were identified with Ponceau S stain (general-purpose protein stain) and bovine serum albumin was used for each run as a standard. Reagents and migration media were manufactured by Helena Laboratories (Beaumont, Tex.). The electrophoresis data provide a measure of purity of the enzyme. A single line corresponds to a single protein mobility, while a band of lines indicates several types of protein or a single protein with a varying amount denaturation.

Factors which determine stability of the free enzyme were investigated early in the program. The effects of NAD, pH, and temperature were investigated. It was soon discovered that oxidation was a primary mode of degradation at room temperature. Storage at pH 7, at room temperature for days at a time, was found to be possible if oxygen was excluded, while bubbling oxygen through a GD solution produced immediate loss of activity.

The effect on enzyme activity and stability of several ions which have been stated as affecting activity was also investigated including NH_4^+ , Na^+ , and K^+ . The temperature dependence of activity was also investigated, as was an agent for protection of SH bonds, dithioerythritol (see "Immobilization Protocol" section on p. 12).

To extrapolate reaction kinetics obtained at one glycerol or NAD concentration with those at another, the Michaelis constants K_m of the free enzyme were estimated. The initial velocity V_o of any first-order enzyme catalyzed reaction is given by:

$$V_o = \frac{C}{C + K_m} V_m \quad (2)$$

where C is the concentration of the reactant in question, e.g., glycerol, and V_m is the maximum velocity attainable. Burton (4) gives K_m for glycerol as 39 $\mu\text{mole/ml}$, while others have given K_m as 2-4 $\mu\text{mole/ml}$.

At concentrations above K_m the velocity is near V_m , while at concentrations much lower than K_m , equation 2 indicates that V_0 is much smaller than V_m and varies linearly with concentration. A typical serum triglyceride level is 100 mg/dL. This level is equivalent to about 10.4 mg glycerol/dL or 1.1 $\mu\text{mole/ml}$. Dilution of the serum for the assay and in a prior lipase hydrolysis step will mean that the glycerol concentration at the time of measurement is likely to be much smaller, on the order of 0.1 $\mu\text{mole/ml}$. Thus the reaction velocity will be well below the maximum and the K_m value is important in estimating reaction velocity. K_m was determined by measurement of initial reaction rate at high fixed NAD concentration with glycerol concentration varying over a wide range: from 0.5 to 400 $\mu\text{mole/ml}$. The K_m for NAD was also measured. The K_m value indicates a lower limit for NAD concentration such that most of the GD will bound to NAD in solution. As discussed in the "Results" section on p. 16, we found a much higher immobilized GD activity with NAD present during immobilization and NAD was present during storage of the immobilized enzyme at a level much greater than the measured K_m .

Enzyme Carrier

Carrier materials were limited to those readily available in tubing form, but which for purposes of the study were utilized in the form of microfibers or microspheres in order to present a large surface area for immobilization. Several basic coupling methods including both physical adsorption and chemical coupling schemes were evaluated. Physical adsorption (no chemical bond) to nylon was evaluated as a representative physical adsorption method. Nylon had been shown to irreversibly bind several blood plasma proteins at our laboratory.

Covalent chemical coupling via well-defined linkages generally involves two steps: a) activation of the carrier surface (incorporation of functional groups into the surface which can chemically combine with groups on the enzyme molecule), and b) the actual covalent attachment. Most of the methods for covalent enzyme immobilization used to date involve chemical coupling to groups characteristic of any amino acid polymer.

One common chemical coupling method used to bind enzymes (7) and other proteins (8) employs a dialdehyde linkage between a free amine group on the carrier and a free amine side chain of the enzyme, usually lysine. The actual covalent coupling is straightforward. Glutaraldehyde has been shown to be satisfactory as the ligand. The key problem is "surface activation" or incorporation

of numerous free amine groups into the carrier. Nylon contains free amine groups but only as end groups of the molecule. Nylon 6,6, the commonly available material, has predominantly COOH end groups and relatively few amine terminations (10). Acid hydrolysis will produce additional end groups, but will degrade the material. Alternatively, amine groups can be incorporated into silicone rubber or glass using a multifunctional silane cross-linking agent containing an alkyl amine chain. Gamma-amino-propyl-triethoxy silane (GAPTES) is one such agent. The ethoxy groups readily hydrolyze to form ethyl alcohol and a trifunctional intermediate having three reactive OH groups. These react with other available OH groups (OH terminations of polydimethyl siloxane, silanol groups on glass, or OH groups from other GAPTES molecules), effectively cross-linking the reactants, with a pendant propyl amine group at the reaction site.

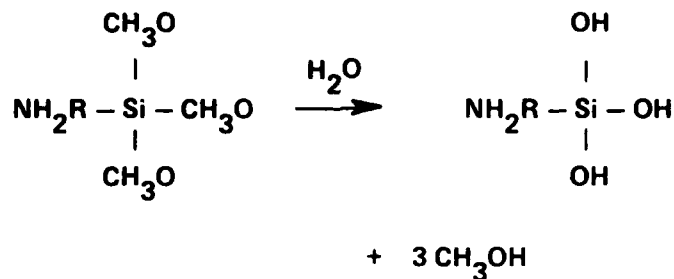
Glass was chosen as the carrier for the propyl amine groups because it is possible to get a very high surface density (Fig. 1), much higher than on silicone rubber where bonding is limited to chain terminations and much higher than on hydrolyzed nylon. In addition, glass microfiber carriers are readily available as are innumerable types of glass tubing.

We also briefly evaluated the use of several multifunctional coupling agents in combination. By using a mixture of GAPTES and a similar coupling agent with nonpolar groups substituted for the amine groups such as methyl triethoxy silane or methyl triacetoxysilane (MTAS), it was possible to systematically vary the hydrophilic/hydrophobic nature of the surface. This also provides a way of simulating amine-activated silicone rubber. The main question to be answered in these experiments was: Does a progressively more hydrophobic backbone between amine groups improve activity of the bound enzyme?

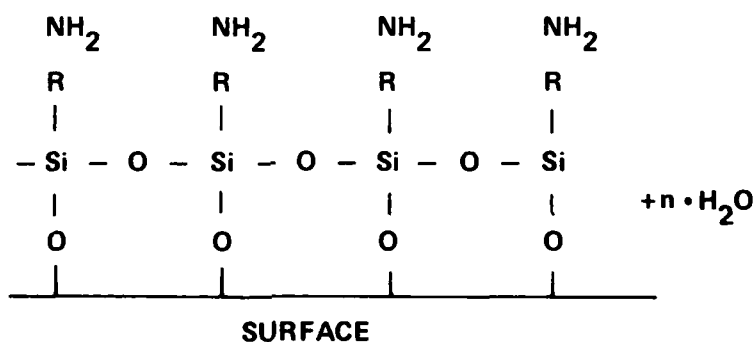
In addition to dialdehyde amine bonding, the amine-glass itself represents a promising coupling surface. At neutral pH nearly all of the amine groups are protonated, producing a positively charged surface with a high density of NH_3^+ groups. These would be expected to have affinity for negatively charged amino acid-side chains such as aspartic or glutamic acid. Despite extensive use of dialdehyde-amine coupling in the literature, no discussion of the amine surface alone could be found. As will be discussed in the "Results" section, this rather simple concept was found to be promising.

In summary, three basic immobilization methods were evaluated:

- a) physical adsorption to nylon 6,6
- b) chemical coupling via glutaraldehyde to GAPTES-glass
- c) coupling to GAPTES-glass with no dialdehyde present.



A. FORMATION OF REACTIVE TRIFUNCTIONAL INTERMEDIATE



B. SELF POLYMERIZATION AND SURFACE ATTACHMENT

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Figure 1. Carrier activation via trifunctional silane. Propylamine groups are coupled to silanol groups of a glass carrier surface by means of GAPTES trifunctional silane.

In addition, binding to glutaraldehyde-GAPTES-MTAS-glass (dialdehyde hydrophobic amine surface) was briefly investigated.

Nylon 6,6 carriers were microspheres averaging about 0.3 μm in diameter and having a specific surface area of about 150,000 cm^2/gm . The microspheres were prepared by a technique developed by the Principal Investigator (unpublished). Briefly, the process involves precipitation of a nylon solution in a turbulent jet to form a fine colloidal suspension.

Borosilicate glass carriers were microfiber filters (Millipore Corp., Bedford, Mass.) containing no resin binder or other impurity. The filters consisted of fibers having diameters in the range of 5-20 μm , and random spacings on the order of 20-50 μm . Each filter weighed approximately 34 mg and had a surface area of about 1300 cm^2 . The surface area was determined by analysis of argon gas adsorption (Brunauer-Emmett-Teller isotherm) at liquid argon temperature.

Incorporation of Active Functional Groups Into the Carrier Surface

Amine groups were coupled to glass carriers using aqueous GAPTES solutions. Glass filter carriers were cleaned in tetrahydrofuran (THF) and immersed in 10% solution of GAPTES in deionized water for 1 hour at elevated temperature, usually about 75°C. Excess solution was drained and the filters were dried at 115°C for 2 hours. The filters were then thoroughly washed with deionized water, dried again, and stored. Typical weight gain of a filter was about 12-15 mg. Nonaqueous solvents for GAPTES, e.g., THF, produced less adherent GAPTES, and were not utilized for the immobilization experiments. GAPTES was obtained from Pierce Chemical Co. (Rockford, Ill.) and Petrarch Systems, Inc. (Levittown, Pa.).

To ensure that surface amine groups were indeed present, the filters were immersed in saline and titrated against 0.1 N HCl. With bare filters, little or no pH change was observed on adding saline. With the amine-activated filters a pH near 10 was initially measured, as would be expected for amine protonation (for propylamine $K_a = 0.25 \times 10^{-10}$). As HCl is added, excess OH^- is neutralized, and all of the NH_2 is converted to NH_3^+ . The titrated amount of HCl directly measures the number of amine groups since excess OH^- is entirely due to amine protonation. Saline provides a large excess of ions and is necessary for the titration, since some negative ions tend to remain at the positively charged surface. Note that this simple characterization of amine-derivatized surface is made possible by the use of very large surface area carriers. With normal laboratory surface areas no effect could be measured.

Some of the amine-glass filters were exposed to 1% glutaraldehyde in potassium phosphate buffer at room temperature for 30-45

minutes. A color change could generally be seen. (The filters turn dark red.) The filters were exhaustively washed in a Wheaton box overnight and stored wet. Representative filters from each batch were titrated with HCl as above, to assay for remaining unreacted NH_2 groups. Generally there was little pH change with a negligible amount of HCl necessary to return to neutral pH, indicating that nearly all of the amine groups had reacted with an aldehyde group of the glutaraldehyde.

It is known that commercial glutaraldehyde can polymerize on storage. This would render the coupling agent less than optimally effective. The polymer is indicated by an absorption peak at 2350 \AA (6). No such absorption peak was ever seen in our glutaraldehyde samples. Nevertheless, scanning electron micrographs of some of our filters indicated small amounts of a gel-like substance in the fiber network. It was also observed that perfusion of glutaraldehyde through a $0.2\text{-}\mu\text{m}$ membrane filter required increasing pressure in time, also indicating the presence of a gel-like substance. Purification of the glutaraldehyde was therefore implemented via ultrafiltration. A $0.01\text{-}\mu\text{m}$ ultrafiltration membrane (Amicon XM-300, Amicon Corp., Lexington, Mass.) was used.

Immobilization Protocol

Immobilization to all carriers was carried out in 0.05 M potassium phosphate buffer (KPB) at pH 7. In most of the procedures NAD was also present at $1.5 \text{ }\mu\text{mole/ml}$. Particles were exposed to the protein for a fixed period of time, usually 20 minutes, and then washed by centrifugation and resuspension in fresh buffer or buffer-NAD. The original supernatant was retained for assay of total protein. The particles were again washed two more times prior to assay or storage.

Filters (amine-glass, glutaraldehyde-amine-glass, and glutaraldehyde-amine-hydrophobic glass) were incorporated in a flow system using ordinary plastic syringe filter holders and a Harvard syringe pump. Nearly all of the filter immobilizations were carried out with NAD present. Immobilization to the filters involved:

- 1) prime with 3 ml NAD-KPB (volume of filter holder + connecting tube - 2.4 ml)
- 2) perfusion of 1.5-ml enzyme solution (0.1 to 0.6 mg/ml GD, 1 mg/ml NAD, in 0.05 M KPB)
- 3) wash with 3 ml NAD-KPB
- 4) collection of all effluents for determination of unbound protein
- 5) thorough wash with 30 ml of NAD-KPB
- 6) storage with NAD-KPB.

It was determined early in the program that nearly all un-bound protein was eluted in steps 2 and 3. Step 5, which involves a wash with 10 times the volume of step 3, has been shown to elute almost no protein. Enzyme present at the end of step 5 is considered to be irreversibly bound. Each assay for activity involves an initial additional wash prior to exposure of the immobilized enzyme to glycerol.

Extension of the time of exposure of the enzyme to 2 hours (steps 2, 3) showed no increase in subsequent activity (in fact, lower activity was measured), and the 20-minute exposure was routinely utilized. An agent for protection of SH groups, dithioerythritol (DTE) was added to the enzyme in some of the runs. DTE is known to split S-S bonds of proteins while protecting SH bonds from oxidation (5). In addition, it has been reported that at 1 mM, 1/10 the concentration usually used for S-S cleavage, DTE enhances GD activity, perhaps by retaining its ability to bind SH groups (9). The effect of this reagent in protecting the enzyme during immobilization was evaluated.

Protein Assays

Protein concentrations of solutions were measured by a modification of the dye binding method of Bradford (3). One ml of stock dye solution (BioRad Laboratories, Richmond, Calif.) was diluted to 5 ml with distilled water. The diluted dye was vortexed with 1 ml of solution, and the absorption spectrum was determined from 5000 Å to 8000 Å in a dual-beam spectrophotometer using the dye solution alone as a reference. The peak at 5900 Å is proportional to the protein concentration over a wide range of concentration, as seen in Figure 2. The data shown were obtained using various concentrations of a bovine gamma globulin standard solution. The linearity at the above protein/dye ratio was better than that claimed by BioRad. The entire spectrum was measured to verify the presence of a peak, and to determine a small background correction off peak at 8000 Å. Using the method, enzyme protein as low as 0.02 mg/ml can be measured. The method is much more sensitive than either UV absorption at 2780 Å or the biuret method. We evaluated the effect of complete protein denaturation on absorbance by repeating a measurement in 6 M urea. Only a 9-10% change in absorbance relative to that seen under standard conditions was observed for the fully denatured protein.

In the immobilization experiments the amount of bound protein is measured by depletion. Protein concentrations are measured before and after surface exposure with corrections made for dilution. The bound protein data are not in absolute units since an exact correlation between absorbance and GD concentration is not known. The relative data are sufficient for analyzing the immobilization procedures. A protein standard (bovine gamma globulin--BGG) was run with each set of enzyme protein determinations to provide a

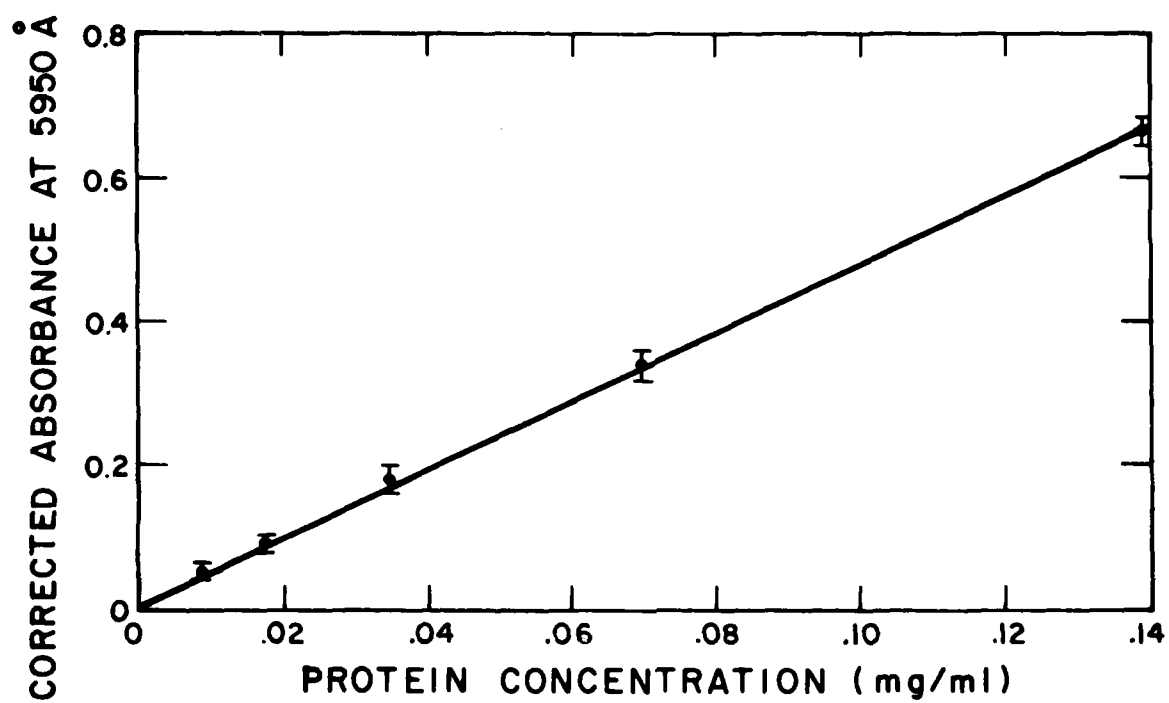


Figure 2. Linearity of protein assay.

consistent control between different sets of enzyme solutions. Generally the absorbance of the BGG standard was constant from batch to batch.

Measurement of Enzymatic Activity

Enzyme activity was routinely measured at pH 9.5 in 0.1 M glycine buffer at 25°C. Activity of the free enzyme was measured by combining: 1.2 ml 1 mM glycerol-buffer solution, 1.5 ml NAD-buffer solution containing 5 mg/ml NAD, and 0.3 ml enzyme-NAD-KPB solution containing 0.01 mg/ml GD. As indicated in equation (1) glycerol is oxidized to dihydroxyacetone while NAD reduced to NADH in the enzyme reaction. The NADH was quantitated in time by measurement of absorptivity at 3400 Å using a dual-beam spectrophotometer with NAD-buffer as a reference.

The activity of enzyme immobilized to particles was measured by resuspending the particles in NAD-buffer and adding glycerol solution for a fixed period of time with the reactants maintained at 25°C. The reaction rate was slowed at the end of the exposure period by reduction of the pH. The particles were then removed via centrifugation, and the absorptivity at 3400 Å of the solution compared with that of a control solution containing a known amount of enzyme, which had processed in exactly the same way.

Most of the activity measurements involved enzyme coupled to microfiber filter carriers incorporated in a flow system as described in the "Immobilization Protocol" section. Just prior to an activity measurement, the carrier was thoroughly washed with 30 ml of NAD-KPB solution. The assay involved further wash with 3 ml NAD-glycine followed by perfusion of the actual reactant mixture. Typically 9 ml of solution containing glycerol and NAD in 0.1 M glycine buffer at pH 9.5 at concentrations identical to those used for the free enzyme assay were perfused through the carrier. The perfusion flow rate, 4.5 ml/minute, was high enough so that the reaction equilibrium was not approached, and the enzyme catalyzed reaction velocity was maximum throughout the perfusion period. The 9 ml of reaction mixture was the volume equivalent of many assays in a typically clinical chemistry analyzer. The middle 4.5 ml of the reacted solution was collected, diluted, and absorptivity at 3400 Å was immediately measured. In addition, the full absorptivity spectrum from 3000 Å to 8000 Å was routinely measured. Any increase in time was also noted as this would indicate that the reaction was proceeding in the cuvette, and that enzyme had been eluted from the carrier during assay.

The first assay of irreversibly bound enzyme activity was always carried out just after immobilization. (Wash step in between of course.) Activity was determined as equivalent weight of enzyme by normalization to a control containing enzyme solution whose protein concentration had been measured and which was run concurrently. Unit surface activity was determined by dividing the normalized activity by the surface area of the carrier:

$$\text{Specific Bound Activity } (\mu\text{g}/\text{cm}^2) = \frac{\text{Measured Carrier } (\mu\text{mole}/\text{min}) / \text{Carrier Surface } (\text{cm}^2)}{\text{Control Enzyme } (\mu\text{mole}/\text{min}) / \text{Control Enzyme } (\mu\text{g})} \quad (3)$$

Expressing activity in terms of equivalent amount of protein per unit surface area of carrier facilitates comparison with the bound protein measurements and, in addition, enables activity in a particular device application to be easily estimated.

Storage and Reassay of Immobilized Enzyme

Experiments to determine whether the immobilized enzyme would retain activity after room temperature storage were begun during the last portion of the 9-month program, once a workable immobilization procedure had been at least partially defined. The microfiber carriers were stored in the filter holders with NAD present, in KPB at room temperature. One mg/ml NAD concentration was used.

Several carriers were reassayed periodically. Prior to each reassay the filter was thoroughly washed by perfusion with 30 ml NAD-KPB solution. Each reassay involved 9 ml of glycerol reactant solution as described in "Measurement of Enzymatic Activity" section, and each filter was again thoroughly perfused with a NAD-KPB rinse prior to storage.

RESULTS

Characterization of Free Enzyme

Activity and Purity of Commercial Preparations-- When assayed as described in "Protein Assays" section on p. 13, the various commercial preparations initially yielded approximately the following activities:

- a) Sigma Laboratories, approximately 2 1/2 U/mg protein.
- b) PL Laboratories, approximately 4 U/mg protein.
- c) Worthington, approximately 4 U/mg protein.
- d) Boehringer Mannheim, approximately 10-20 U/mg protein.

Only the Boehringer Mannheim sample was deemed satisfactory, and this supplier was used exclusively during most of the program. (Several samples of both PL and Sigma enzyme were evaluated, while only one batch of the Worthington enzyme was evaluated. Our object was simply to select a supplier of consistently high activity glycerol dehydrogenase.) When an inhibiting ion, Na^+ , was removed from the buffer, various batches from Boehringer Mannheim generally

yielded approximately 25 U/mg, based on both dilution of stated protein contents of an aliquot, and the actual protein assay.

Protein electrophoresis of the Boehringer Mannheim product revealed a single line having a mobility approximately equal to that of the serum albumin control. Electrophoresis of the Sigma sample revealed a line at nearly the same position--at the albumin mobility. However, a set of diffuse bands trailing back nearly to the origin were also seen, suggesting an impure preparation consisting of a multitude of different species.

Factors Which Affect Activity and Stability of the Free Enzyme--The principal factor affecting stability of the enzyme in solution was found to be oxidation. It was found early in the program that storage of enzyme solutions at 4°C was not necessary if air in the storage vessel was excluded. The enzyme could be stored for several days at room temperature at pH 7 with little or no loss in activity. On the other hand, bubbling oxygen through a GD solution produced immediate loss of activity. Figure 3 shows spectrophotometer tracings of absorbance at 3400 Å vs. time for two enzyme solutions, one of which had oxygen bubbled through it a few minutes prior to the assay. The loss of activity is pronounced.

Optimal stability of the enzyme at room temperature storage was achieved in KPB-NAD solution with air excluded. Loss of activity occurred at pH 9.5 in glycine and in glycine-NAD. The NAD concentration in all of the solutions was 1 mg/ml.

Na^+ was found to inhibit activity by about 1/3 when present in the buffer solutions (typically at 0.04 M concentrations). All buffer solutions were brought to the desired pH with KOH rather than NaOH after this effect was discovered.

NH_4^+ is listed as an activating ion by the manufacturer (Boehringer Mannheim). The GD is received as a 20 mg/ml suspension in 3.2 M ammonium sulphate solution. At 0.4 mg/ml, a typical concentration in an adsorption experiment, NH_4^+ is present at about 0.06 M. It was not necessary to add this ion to the reactants. The PL and Sigma preparations were lyophilized with no NH_4^+ present. When these preparations were assayed in glycine buffer, the addition of NH_4^+ was not found to affect activity.

Dithioerythritol at 1 mM had no effect on activity of the free enzyme, in contrast to the results in reference 9.

The effect of temperature is shown in Figure 4, where NADH production (absorbance at 3400 Å) is plotted vs. time for reactant solutions at 3 temperatures. The data indicate an increase in activity of about 25% for a temperature increase of 5°C. The activity at 20°C was reduced by about 23% from that at 25°C, while that at 30°C increased by about 27%.

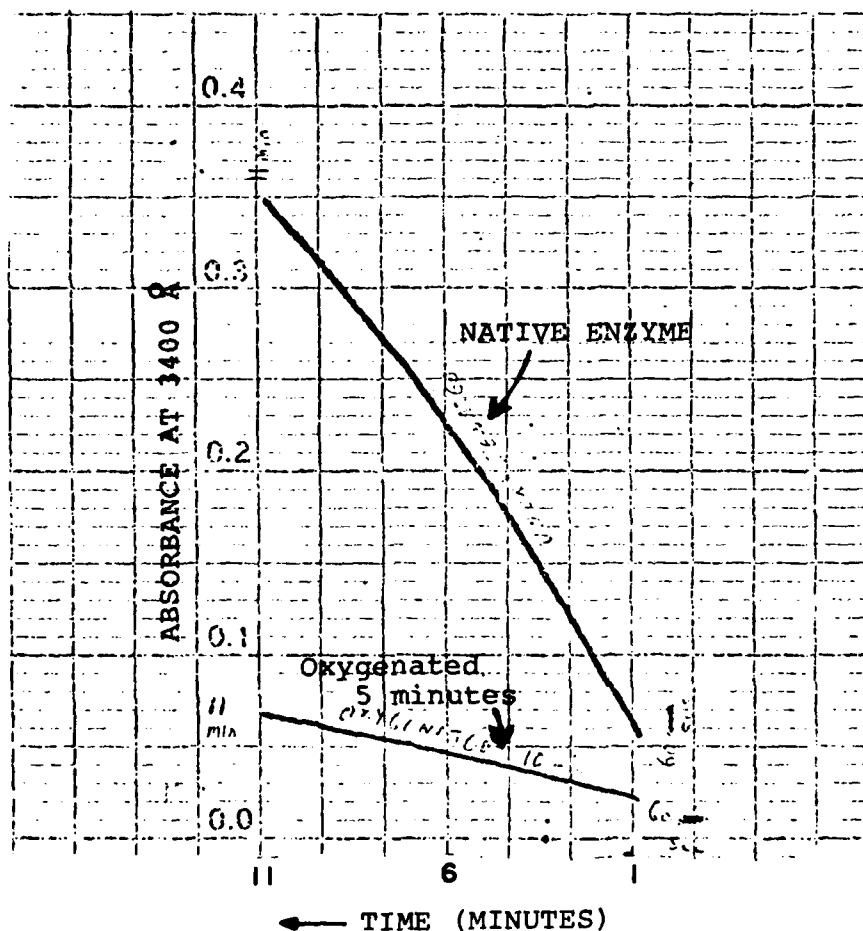


Figure 3. Reaction rate for native and oxygenated enzyme solutions. Spectrophotometer output as recorded shows time evolution of NADH production for two enzyme reaction solutions, one of which had been bubbled with oxygen, prior to the addition of glycerol. Loss of enzymatic activity by the oxygenated solution is apparent.

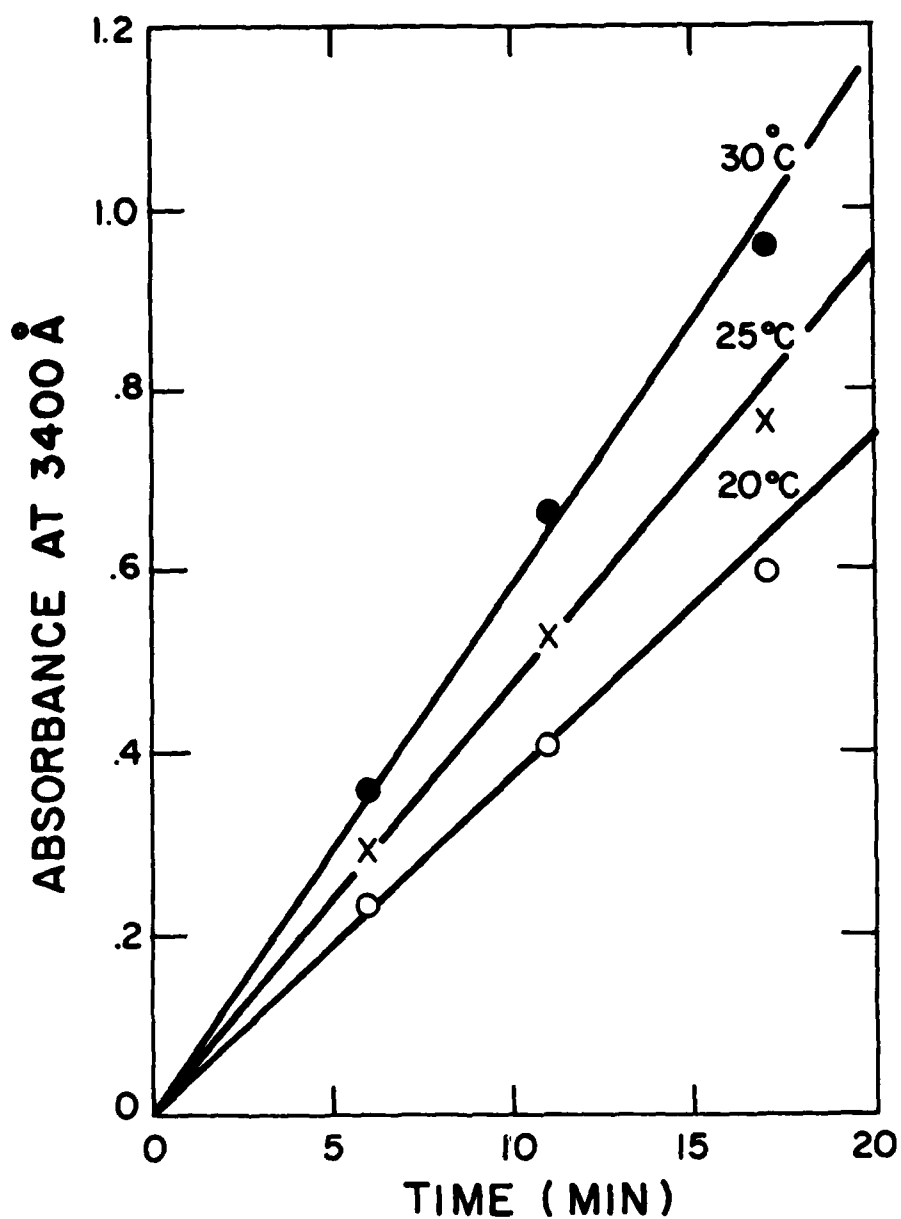


Figure 4. Temperature dependence of glycerol dehydrogenase activity. NADH production is indicated vs. time for reactant solutions thermostated at 20°, 25°, and 30°C. The reaction rate increased by about 25% for each 5°C increase in temperature.

The kinetic constants were briefly investigated as described in "Characterization of Free Enzyme" section on p. 6. The initial velocity data approximately fit the Michaelis Menton equation for glycerol concentrations in the range 1 to 100 μ mole per ml, with the best fit at $K_m = 1.85 \mu$ mole/ml. At glycerol concentrations much larger than this value, the initial reaction velocity saturated at the maximum rate (see Figure 5). At 400 μ mole/ml (off scale on the plot) the initial velocity was higher than the saturation value shown by about 60%. An additional glycerol-NAD-GD binding mechanism would seem to be indicated at very high glycerol concentration. Investigation of further details of the kinetics was beyond the scope of this study.

Kinetic data for NAD are shown in Figure 6. The plot fits the Michaelis-Menton form, over the entire range investigated from 0.12 μ mole/ml to 5.6 μ mole/ml. The data yield a K_m of about 0.25 μ mole/ml. Maximum reaction velocity is achieved at NAD concentrations much larger than this value. The 1 mg/ml NAD used for storage of immobilized carriers is equivalent to 1.5 μ mole/ml, at which concentration the percent saturation (percent of enzyme bound to NAD) is about $1.5/(1.5 + 0.25)$ or 86%.

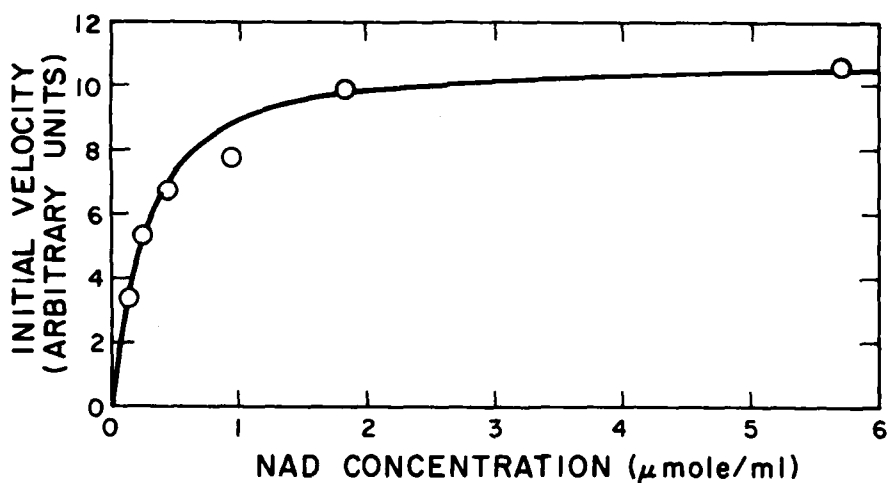
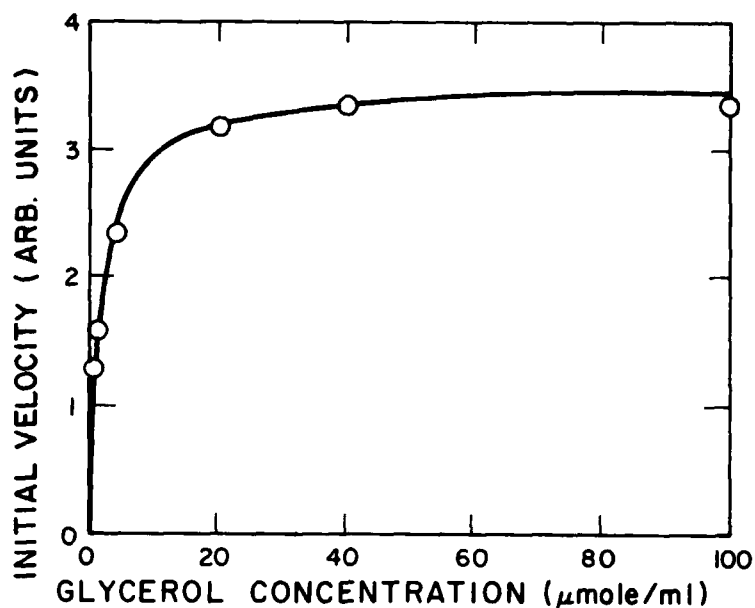
The above kinetic constants are in the same range as those obtained by others:

	<u>This study</u>	<u>Barrett (2)</u>	<u>McGregor (9)</u>	<u>Burton (4)</u>
K_m (NAD) (μ mole/ml)	0.25	0.28	0.20	0.15
K_m (glycerol) (μ mole/ml)	1.85	2.4	1.25	39

The other studies referred to each used a different enzyme preparation and a different buffer system. All were carried out near pH 9 with activating ions present if such were required. The free enzyme, as assayed by us, was thus found to have kinetic constants which were close to those observed by others.

Characterization of Carriers

Surface Area--The nylon particle carriers had a specific surface area of 150,000 cm^2/gm and were used without surface activation in physical adsorption experiments. Most of immobilization involved coupling to activated microfiber glass carriers. The glass fiber filters had 1300 cm^2 surface area per filter, and each filter weighed 34 ± 2 mg. The fiber medium had a surface area of 3.9 m^2/g , as determined by argon gas adsorption.



Figures 5 and 6. Glycerol dehydrogenase kinetics. Figure 5 shows dependence of initial reaction velocity V_0 on glycerol concentration for NAD fixed at 5.6 $\mu\text{mole/ml}$. The solid curve shows the theoretical Michaelis-Menten curve with $K_m = 1.85 \mu\text{mole/ml}$, while the O's indicate experimental points. Figure 6 shows analogous data for the NAD substrate with fixed glycerol concentration. A good fit is seen between experimental points and the Michaelis-Menten form with $K_m = 0.25 \mu\text{mole/ml}$ (solid curve).

Surface Activation--Titration assay of amine group activation consistently showed no effect on the bare filters, while the GAPTES-treated filters required approximately 1.4 ml of 0.1 N HCl for neutralization of OH^- generated by protonation of the surface amine groups on one filter. Thus the GAPTES-treated carriers had $1.4 \cdot 10^{-4}$ moles of NH_3^+ groups per filter. This is equivalent to about $6.5 \cdot 10^{16}$ groups per cm^2 , a very dense surface coverage, probably indicating multiple GAPTES layers. The molar coverage per filter as measured by titration can be compared to the measured weight gain by multiplying the number density of NH_3^+ groups by the molecular weight of the silane molecule after undergoing the surface reaction (101 atomic mass units). This yields 14 mg which approximates the measured weight gain of the filters during activation.

The glutaraldehyde-treated filters produced a rise in pH but required a negligible amount of HCl for neutralization, generally about 0.02-0.04 of the amount used for GAPTES filters prior to glutaraldehyde treatment. The glutaraldehyde treatment was thus directly shown to have reacted with about 96-98% of the available surface amine groups of the carriers.

Results of Immobilization Procedures

Adsorption to Nylon--Data for amount of bound protein and specific bound activity are shown in Table 1 for two initial enzyme concentrations for two adsorption conditions: enzyme adsorbed in KPB at pH 7.2, and enzyme adsorbed in KPB with added $1.5 \mu\text{mole/ml}$ NAD. The presence of NAD during adsorption does not affect the amount of bound enzyme, but clearly has a dramatic effect on the activity of the bound enzyme, increasing it by an order of magnitude. The data were obtained with 15 mg of particles/ml, equivalent to about $2200 \text{ cm}^2/\text{ml}$. The data indicate a small specific bound activity, of the order of $0.01 \mu\text{g}/\text{cm}^2$. Greater than 50% of the bound protein is active, a rather striking result, but the total amount of bound protein seems to be too small to be of practical interest. The principal result of these experiments is the role of NAD in preserving activity during adsorption.

Coupling to Amine-Glass: Adsorption to Charged Amine Groups, and Chemical Coupling via Dialdehyde-Amine Linkage--The two types of coupling to amine-glass are discussed together, as they have many features in common. Data for coupling directly to amine glass (GAPTES-activated glass) carrier are given in Table 2, while data for coupling to glutaraldehyde-amine glass carrier are given in Table 3. Shown in each table are: the enzyme solution concentration during immobilization, the amount of bound protein in micrograms per filter carrier, and the surface activity per filter carrier expressed as an equivalent weight of free enzyme.

The results for each type of coupling were dependent on the source of GAPTES, and the data are presented in two groups:

- 1) denotes carriers prepared using GAPTES from Pierce Chemical Co.
- 2) denotes carriers prepared in exactly the same fashion using GAPTES from Petrarch Systems, Inc.

Carriers treated with GAPTES silane activating agent from each manufacturer yielded nearly the identical surface density of amine groups as measured by titration.

Direct coupling to amine-glass (group 1) carriers (GAPTES (group 1)) yielded substantial surface activity which increased with solution concentration up to a maximum of about 110-120 μg equivalent weight. As described in "Measurement of Enzymatic Activity" section on p. 15, surface activity is measured after thorough washing of the treated surface with NAD-buffer and represents activity of irreversibly bound protein as measured shortly after immobilization. For GAPTES (group 1) carrier an initial enzyme concentration of 0.123 mg/ml yielded 66 μg equivalent activity, a solution of 0.28 mg/ml yielded 88 μg , and solutions in excess of 0.4 mg/ml yielded equivalent activities in the range of about 110-120 μg . (See Table 2.) This activity for the 1300 cm^2 filter carrier is equivalent to about 0.09 $\mu\text{g}/\text{cm}^2$ on a unit surface area basis.

Glycerol dehydrogenase coupling via glutaraldehyde linkage to the same carrier yielded nearly identical activity at low solution concentration, and similar activities at higher concentration. The value 152 indicated in the table includes some weakly bound enzyme which eluted from the surface during the first assay at pH 9.5. Upon reassay, the carrier activity was consistently in the range of 100 to 110 μg .

Both direct and dialdehyde coupling to GAPTES (group 1) activated carriers resulted in substantial initial activity, in excess of 100 μg per filter. Total bound protein fluctuated from run to run, but was about 200 to 250 μg for the high-activity filters. Neither surface coupled all of the available enzyme, and somewhat surprisingly several of the filters retained activity for substantial periods of time. Retention of activity in time involved re-assay after storage in NAD-KPB at room temperature, with no air present. Several of the filter holders developed leaks and the enzyme became inactivated, and were not assayed further. Retention characteristics of the GAPTES (group 1) carriers (no glutaraldehyde) showed gradual deterioration in time. A typical carrier lost about 10-15% activity per week upon storage. Several of the dialdehyde-coupled GAPTES (group 1) carriers showed retention of activity at a constant level for several weeks as shown in Figure 7. Note that the stability of NAD is limited, and free GD is also kept at 4°C just prior to assay in published protocols, so that the observed stability over 4-5 weeks at room temperature is all the more remarkable.

TABLE 1. THE EFFECT OF NAD ON THE ACTIVITY OF GLYCEROL DEHYDROGENASE ADSORBED TO NYLON

Glycerol dehydrogenase concentration		Adsorbed protein	Activity of bound enzyme*
<u>Initial</u>	<u>Equilibrium</u>		
<u>NO NAD</u>			
120 µg/ml	88 µg/ml	32 µg	2.1 µg
240 µg/ml	190 µg/ml	50 µg	3.3 µg
<u>NAD</u>			
120 µg/ml	82 µg/ml	38 µg	21 µg
240 µg/ml	192 µg/ml	48 µg	25 µg

* Activity expressed as equivalent weight of control.

TABLE 2. BOUND PROTEIN AND SURFACE ACTIVITY OF GLYCEROL DEHYDROGENASE ADSORBED TO GAPTES-ACTIVATED CARRIERS

Surface activation	Protein initial	Conc. ($\mu\text{g/ml}$) equilibrium	Bound protein (μg)	Surface activity equivalent (μg)
GAPTES (1)	123	86	56	66
GAPTES (1)	282	221	92	88
GAPTES (1)	434	266	251	117
GAPTES (1)	492	354	207	93
GAPTES (1)	505			116
GAPTES (2)	449	73	562	138
GAPTES (2)	466	70	590	165
GAPTES (2)	523	10	774	189
GAPTES (2)	573	74	710	221
GAPTES (2)	673	93	870	138
GAPTES (2)	699	146	830	184
Bare glass (for comparison)	607	587	29	12

UNIT SURFACE EQUIVALENT OF ABOVE: $100 \mu\text{g} \rightarrow 0.08 \mu\text{g/cm}^2$
 $200 \mu\text{g} \rightarrow 0.15 \mu\text{g/cm}^2$

TABLE 3. BOUND PROTEIN AND SURFACE ACTIVITY OF GLYCEROL DEHYDROGENASE COUPLED VIA GLUTARALDEHYDE TO GAPTES-ACTIVATED CARRIERS

Surface activation	Protein initial	Conc. ($\mu\text{g/ml}$) equilibrium	Bound protein (μg)	Surface activity equivalent (μg)
GLUT-GAPTES (1)	123	65	87	72
GLUT-GAPTES (1)	282	111	256	83
GLUT-GAPTES (1)	434	261	260	103
GLUT-GAPTES (1)	492	167	437	152
GLUT-GAPTES (1)	607	511	154	72
GLUT-GAPTES (2)	141		211	46
GLUT-GAPTES (2)	282		422	97
GLUT-GAPTES (2)	466		668	131
GLUT-GAPTES (2)	505		372	206
GLUT-GAPTES (2)	562		844	162
GLUT-GAPTES (2)	673		922	193
GLUT-GAPTES (2)	673		999	106
GLUT-GAPTES (2)	699		828	198
GLUT-GAPTES (2)	846		976	255

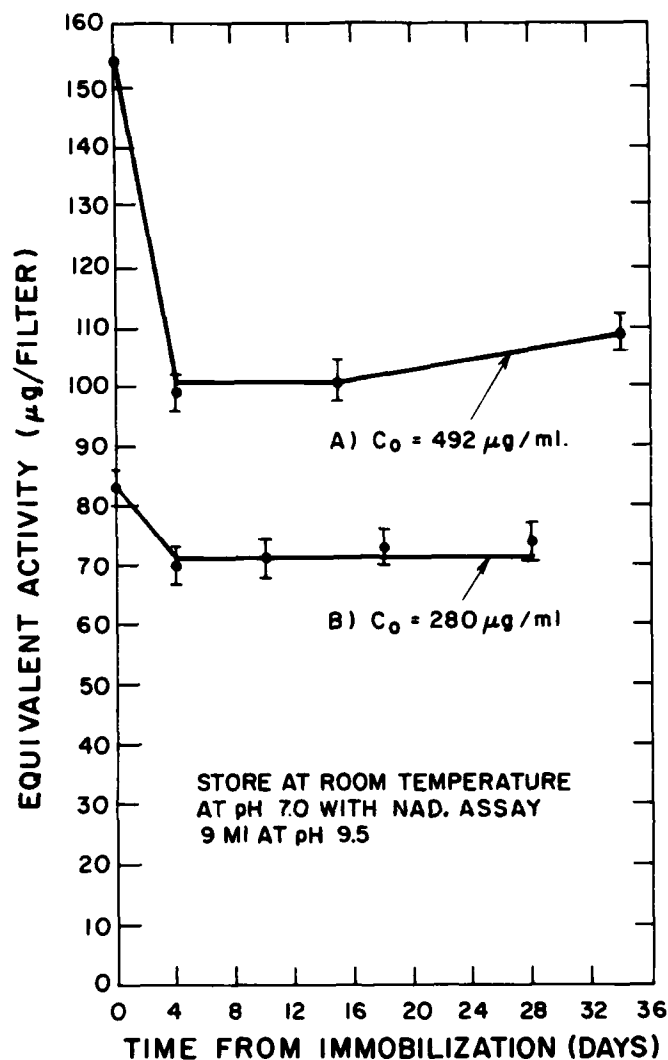


Figure 7. Retention of enzymatic activity by glutaraldehyde-coupled amine glass group 1 carriers. The upper trace (A) shows retention of activity over a 5-week period with carrier storage at room temperature. At the first assay some weakly bound enzyme was eluted, after which enzymatic activity was maintained at a constant level. The slight rise in activity in the last measurement is due to an increase in NAD concentration used in the assay. The enzyme solution concentration at immobilization for this carrier was 0.49 mg/ml. Trace (B) shows retention of activity of a second carrier immobilized with a lower enzyme concentration 0.28 mg/ml.

Results with GAPTES (group 2) activated carriers showed an entirely different pattern than that seen with the GAPTES (group 1). For both direct-coupling and glutaraldehyde-linked enzyme, much higher levels of bound protein and higher surface activities were obtained. The glutaraldehyde-treated (group 2) carriers of this type retained nearly all enzyme protein to which they were exposed in contrast to glutaraldehyde-treated (group 1) carriers which generally retained less than half of the enzyme protein to which they were exposed. Initial surface activities on the order of 200 μg equivalent were observed at protein concentrations greater than 0.5 mg/ml. This activity was not retained over time, however. Typically more than one-half of the initial activity would be lost after a few days at room temperature. A satisfactory method for retaining activity for these carriers (GAPTES (group 2) and glutaraldehyde - GAPTES (group 2)) was not determined during the study. Both the group 1 and group 2 surface treatment yielded high-surface density of amine groups of the same magnitude. The difference between the two sets of activated carrier probably reflects a difference in structure of the silane matrix at the glass surface, perhaps pre-gelling of the Petrarch silane.

It should be noted that the glutaraldehyde-GAPTES (group 1) carriers yielded substantial surface activity, greater than 100 μg per filter, which generally was retained at room temperature storage, and thus a promising immobilization scheme has been identified.

As discussed in the "Procedure and Methodology" section on p. 6, several alterations in the immobilization procedure were briefly examined. Dithioerythritol at 1 mM concentration inhibited immobilization, including reduction in both amount and activity of the bound enzyme. Reassay of the same carrier without DTE showed an increase in activity but not up to the level of enzyme immobilized with no DTE present. DTE even at low 1 mM concentration was not useful for enzyme protection during immobilization. Its effect on retention of activity during storage was not evaluated.

A somewhat more hydrophobic amine glass was also briefly evaluated, and compared with the standard amine glass. A lower density of surface amine groups was present on the more hydrophobic glass, which had been activated with a mixture of GAPTES and methyl triacetoxysilane (MTAS). As discussed, MTAS results in generation of surface methyl groups in place of the propylamine groups produced by GAPTES surface reaction. The measured amine group density was about half of that of the carriers treated with GAPTES alone. The resulting glutaraldehyde-coupled enzyme carrier showed about 78 μg active bound enzyme, about 9% more than a similar glutaraldehyde - GAPTES (group 1) carrier coupled with the same enzyme solution on the same day. Upon reassay after 8 days of room temperature storage the bound enzyme activity was equivalent to approximately 71 μg . These results are not significantly different from the normal GAPTES (group 1) - glutaraldehyde results and

the GAPTES - MTAS treatment was not pursued further. In addition, for comparison, the bare glass filter was evaluated for amount and activity of irreversibly adsorbed enzyme. The bare carrier bound only 29 mg protein, of which approximately 12 mg was active. Bare glass does bind some enzymes irreversibly, but not in sufficient quantity to be useful.

APPLICATION TO DEVELOPMENT OF REACTOR TUBES

General Considerations

Immobilization of glycerol dehydrogenase with retention of activity for at least several weeks has been demonstrated with activity equivalent to about $0.1 \mu\text{g}/\text{cm}^2$ of carrier. The immobilization procedure described on p. 12 is easily adaptable to glass carriers in any form desired. Total enzymatic activity clearly depends on the surface-to-volume ratio of the assay system. If implemented on a filter of the type used, which is then incorporated in a flow system, rapid assay is clearly feasible. The filter would have to undergo a wash cycle between samples.

Implementation as a small bore reactor tube to be retrofit into an existing type of automated flow system analyzer would also be possible, with the resulting activity dependent on the surface-to-volume ratio of the tube. The equivalent enzyme concentration C for a tube of diameter D would be:

$$C (\mu\text{g}/\text{cm}^3) = \frac{40 (0.1 \mu\text{g}/\text{cm}^2)}{D (\text{mm})} \quad (4)$$

At 25 U/mg and 25°C, a 1-mm inner diameter tube would bind the equivalent of 0.004 mg or 0.1 U per ml of solution.

Residence time in the reactor would depend on how close to equilibrium the reaction is to be run. Approaching equilibrium for the glycerol oxidation reaction would require a very long residence time but which would give a result which would be independent of the level of enzymatic activity of the tube and would require relatively few calibration standards to be run. On the other hand, very short residence times would make the observed absorbance dependent on enzymatic activity of the tube rather than the level of glycerol in the sample. As the residence time increases, the absorbance depends on both factors, making the run of calibration standards necessary. An optimum time is one such that for activity within a fixed range, the reaction comes close enough to equilibrium for concentration to be determined from absorbance.

Solution concentrations of glycerol on the order of 0.1 μ mole/ml would be expected. Under these conditions a condition close enough to equilibrium for measurement can be reached in about 15 minutes, based on running the reaction with free enzyme at 0.004 mg/ml. This procedure would allow for substantial throughput since many samples could be reacted in the flow system simultaneously.

Considerations of equilibrium indicate that under the conditions of the assay: a) the observed absorbance will be a nonlinear function of the glycerol concentration, and b) substantially less than 100% of the amount of glycerol in solution will be oxidized. Some of the details of equilibrium considerations are discussed below.

Analysis of Equilibrium

Details of the relation between glycerol concentration and measured absorbance can be determined by analysis of the equilibrium equation. We find that under assay conditions (only glycerol and NAD initially present), equilibrium is determined by two parameters: the ratio of initial NAD to glycerol concentration, and the product of the equilibrium constant K_{eq} and 10^{pH} . Two limiting cases are of special interest. For the condition:

$$K_{eq} 10^{pH} \frac{[NAD]_0}{[G]_0} \gg 4 \quad (5)$$

the reaction will go nearly to completion and the absorbance (NADH production) will be proportional to the total amount of glycerol in the sample. For the opposite case:

$$K_{eq} 10^{pH} \frac{[NAD]_0}{[G]_0} \ll 4 \quad (6)$$

equilibrium will involve much less than complete glycerol oxidation, and the observed absorbance will be proportional to the square root of the glycerol concentration:

$$[NADH] = \sqrt{K_{eq} 10^{pH}} \sqrt{[G]_0} \quad (7)$$

Using $5 \cdot 10^{-12}$ M for K_{eq} (Burton) (4) at pH 9.5 with $[NAD]_0 = 5$ μ mole/ml, $[G]_0 = 0.1$ μ mole/ml, the expression on the left side of the equation 5 is approximately 0.8. Thus equation 5, the condition

for complete glycerol oxidation, is not satisfied. A detailed analysis yields about 57% for the fraction of glycerol oxidized at equilibrium. This percentage can be made higher by running at higher pH, which in our experience reduces activity, or by using a higher NAD concentration. Further dilution of the glycerol solution also would increase the fraction of glycerol oxidized, and result in a more linear response. Dilution is limited by loss in absorbance signal intensity, resulting in a less precise measurement. Thus, a nonlinear relation between measured absorbance and glycerol concentration is implied.

Conclusion

This feasibility study has identified a process for immobilizing glycerol dehydrogenase to amine-activated glass carriers with surface activity equivalent to about $0.1 \mu\text{g}$ of enzyme/ cm^2 . The process can be used for glycerol quantitation with the measured NADH absorbance a nonlinear function of the sample glycerol concentration. The immobilization process is most adaptable to a system with high surface-to-volume ratio. If applied to a reactor tube in a flow system, a small bore tube with residence time of about 15 minutes is desired. Substantial throughput is possible by assaying many samples simultaneously within a single tube.

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